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THE DEVELOPMENT OF THE VASCULAR SYSTEM IN QUAIL EMBRYOS: A COMBINATION OF MICROVASCULAR CORROSION CASTS AND IMMUNOHISTOCHEMICAL IDENTIFICATION

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Abstract

Although vascular casts, obtained by injection with methacrylates, are frequently used to investigate the adult vascular system, little data are available for embryonic stages. In this paper we use Mercox^R in quail embryos in the period of 2 to 7 days after incubation. The microvascular corrosion casts were evaluated in the scanning electron microscope (SEM) with special attention to the development and remodelling of the large arteries and veins. Our results show that the remodelling of the large arteries and veins together with their developing tributary vessels can be visualized from very early embryonic stages onwards. However, complete replication of a developing vascular system depends on diameter and regularity of the lumen. In the stages investigated, the vascular lumen, even of the largest vessels, is still very irregular. Detailed cellular characteristics like nuclear impressions of endothelial cells, as often seen in adult material, were seldom found in the embryos. To examine whether blind-ending sprouts are completely or incompletely replicated in a developing vascular system, additional series of quail embryos were stained immunohistochemically with a monoclonal antibody (MB1) specific for endothelial and hemopoietic cells. It seems that a plexus consisting of endothelial precursors (endothelial cells lacking a lumen) is present in the developing organ before the formation of a lumen and assembly into vessels, which are connected to an adjacent artery or vein. Expansion of the vascular system may in part be due to incorporation of these endothelial precursors in the wall of existing vessels.

Key words: Angiogenesis, antibodies, embryos, endothelium, quail, scanning electron microscopy, vasculogenesis.

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Introduction

The last decade has seen more frequent application of the microcorrosion casting technique in combination with scanning electron microscopy to investigate the vascular system. Most of the investigators studied the vascular system in adult or postnatal tissues, e.g. the vascularization of the vertebral column (Konerding and Blank 1987), lung (Caduff et al. 1986; Schraufnagel 1989; Schraufnagel and Schmid 1988), esophagus (Aharinejad et al. 1989), stomach (Imada et al. 1987), uterus (Kardon et al. 1982) and testis (Murakami et al. 1989). Only a small number of studies concerned the development of the vascular system during the embryonic and fetal period. Burton and Palmer (1989) investigated the chorioallantoic membrane of chick embryos, 6 to 10 days after incubation. The development of the renal vascular system in chick embryos has been described by Ditrich and Splechtna (1989). Sumida (1988) studied light microscopically methacrylate casts of abnormal aortic arches of 12 to 16-day-old rat embryos, which were treated with bisdiamine. Bockman and coworkers (1989, 1990), studying the abnormal development of the pharyngeal arch arteries after ablation of the neural crest, showed some micrographs of vascular casts of 3 to 5-day-old chick embryos.

In their discussion with the reviewers, Ditrich and Splechtna (1989) remarked that it is almost impossible to fill the arterial blood vessels of chick embryos younger than 6 days. Because of our interest in the embryonic development and changes of the vascular system (DeRuiter et al. 1989, 1990A, 1990B), we have used Mercox^R with a modified injection method in avian, specifically quail embryos. Special attention was paid to casts of budding vessels. To study developing vessels with the microcorrosion casting technique, it is necessary to examine whether the casts represent complete or incomplete replications of the vascular system. Therefore additional series of quail embryos were incubated with MB1, a species-specific monoclonal antibody against endothelial

precursors and endothelial and hemopoietic cells (Péault et al. 1983). Moreover, the combination of these two techniques has the advantage to study the relation between the formation of a lumenized vascular system from endothelial precursors and the possibility of blood flow in a developing organ.

Materials and Methods

Vascular casts and SEM

Quail embryos (*Coturnix coturnix japonica*) of 2 to 7 days' incubation were used in this corrosion casting study. The quail embryos were removed from the yolk sac with iridectomy scissors and transferred to phosphate buffered saline (PBS), pH 7.2. The serosa and amnion were removed.

The intra-embryonic vascular resistance of the venous system is lower than that of the arterial system. To prevent the lack of arterial filling, the Mercox (Mercox CL-2B with catalyst MA; Fa Okenshoji Co., Ltd, Chou-ku, Tokyo 104) was injected into the outflow tract of the heart. The low venous pressure of embryos older than 3 days was increased by ligating the vitelline veins. In embryos younger than 3 days, damage of yolk sac vessels should be avoided. To prevent overpressure, leakage was induced in the tail vessels. Glass needles (tip diameter: 4 to 20 μm) were mounted to disposable needles (21G), which in turn were connected to a disposable 1 ml syringe. Because the fragile, immature vessels could easily be ruptured by high pressure, the filling had to be controlled under the dissecting microscope. A small amount, 100 to 200 μl Mercox, was mixed with 1% catalyst, which is recommended by studies on adult material.

The site of injection varied with the stage of development. Embryos with a looped heart (2-3 days) and those without a complete ventricular septum (3.5 to 4 days) were injected into the outflow tract of the ventricle, while older embryos (quail 5 to 7 days) were injected in one of the ventricles. At each age about 7 successful casts were produced.

After injection, the embryos, still in PBS, were placed in an incubator at 37°C for 3 hrs for completion of the polymerization. The tissue was digested in 10% potassium hydroxide solution for 5 to 15 min (37°C). The casts were rinsed several times with distilled water, air dried, mounted on scanning stubs and sputter-coated with gold for 3 min (Balzers MED 010). The microvascular corrosion casts were studied in the scanning electron microscope (Jeol SE 5103 or Philips SEM 525M).

Immunohistochemistry

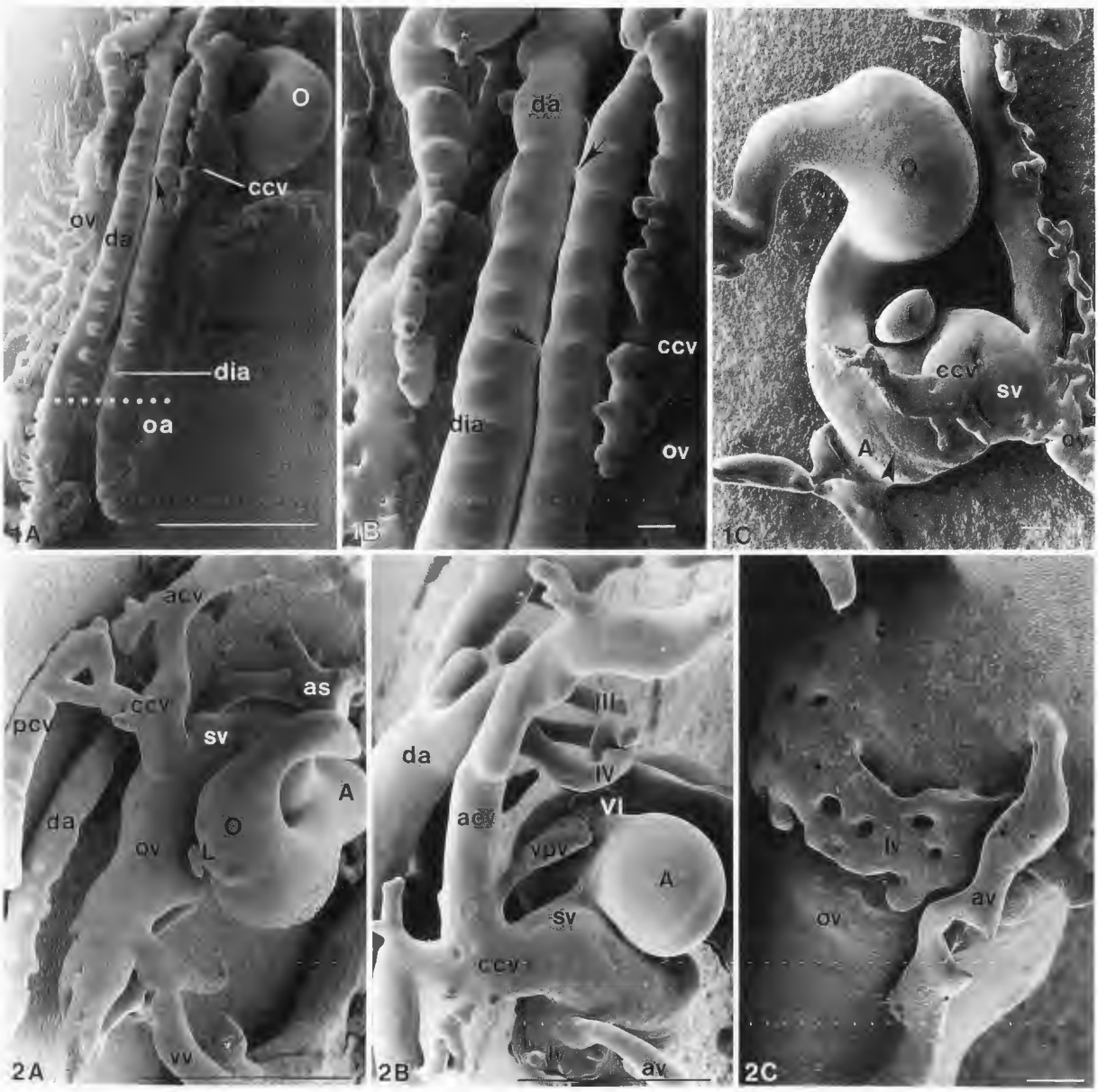
Quail embryos, 2 to 6 days after incubation, were fixed for 24 hrs in periodate-lysine-paraformaldehyde fixative (McLean and Nakane 1974) at 4°C. After embedding in paraplast, the embryos were serially-sectioned transversely at 3μm. The deparaffinated and rehydrated sections were incubated with MB1 monoclonal antibody, which detects quail endothelial cells (Péault et al. 1983), diluted in PBS with 0.05% Tween-20 and 0.1% BSA. The overnight incubation at room temperature was followed by washing in PBS with 0.05% Tween-20. Hereafter the slides were incubated for 2 hrs with the second antibody Rabbit anti-Mouse conjugated to Horse radish peroxidase. After washing in PBS, the staining reaction was performed with 0.04% Diamino benzidine tetrahydrochloride in 0.05M Tris-Maleic acid (pH 7.6) with 0.07% Imidazole and 0.06%% Hydrogen peroxide for 10 min, followed by washing in buffer. Negative controls omitting MB1 and/or the second antibody were also included in the staining reaction. Lastly, the sections were briefly counterstained with Mayer's hematoxylin.

Results

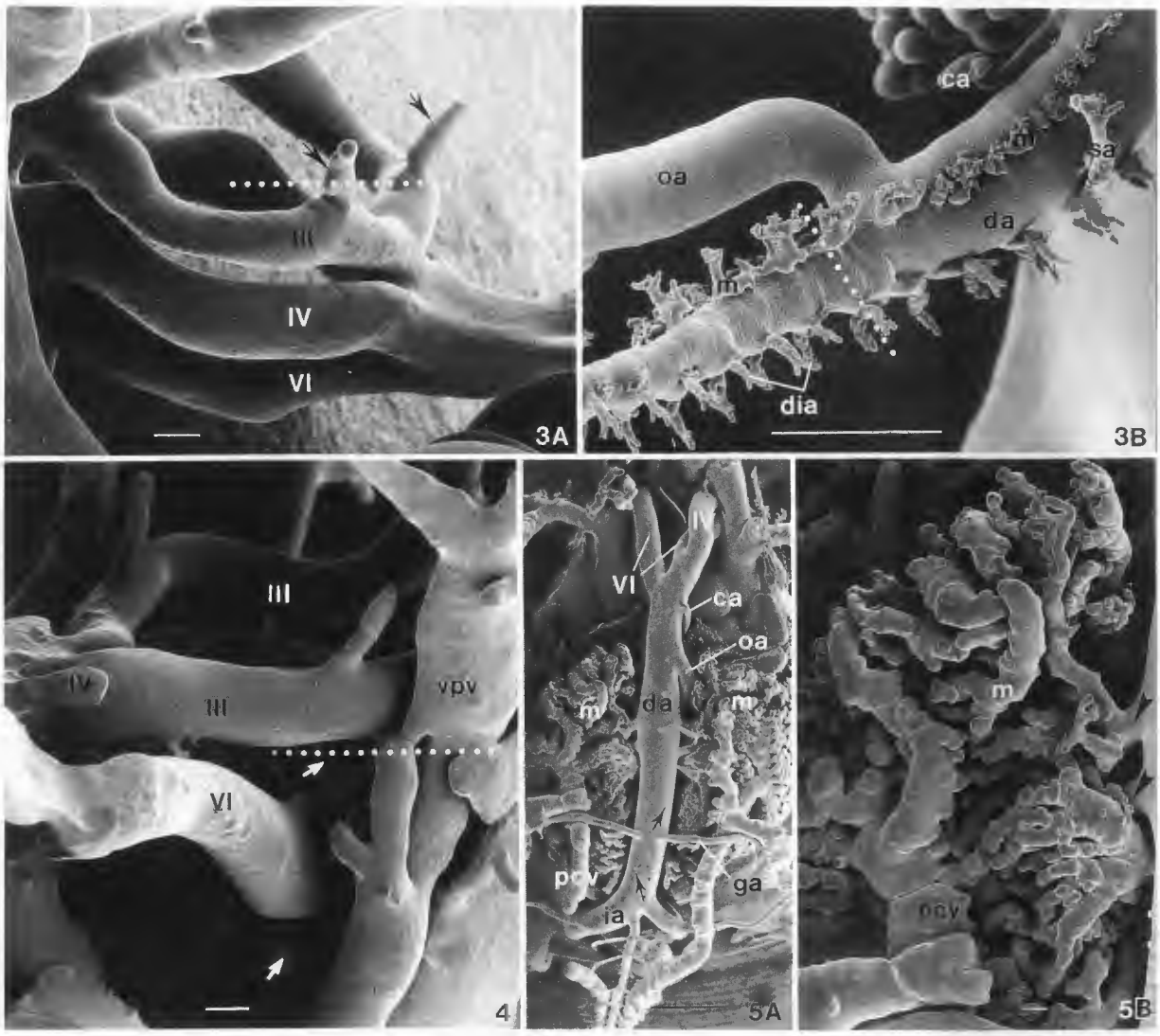
SEM of the vascular casts

In vascular casts of 2-day-old embryos, two dorsal aortae are present. At the cardiac level, the first local fusion as small left-right connections between the paired arteries, are present (Figs.1A and 1B). In the caudal part of the embryo, both dorsal aortae diverge laterally to blend into a plexus of small vessels, which will give rise to the omphalomesenteric arteries. The omphalomesenteric arteries communicate with the vitelline vessels of the yolk sac, which drain into the omphalomesenteric veins. In none of the 2-day-old embryos could the dorsal intersegmental arteries be completely injected, but for a small part at their origin from the dorsal aortae. The proximal part of the posterior cardinal veins, draining this intersegmental system, is filled retrogradely (Fig.1B). In an early 2-day-old embryo the posterior and anterior cardinal veins drain through the common cardinal vein in the omphalomesenteric vein (Fig.1B), but at the end of the second day the common cardinal vein and the omphalomesenteric vein drain separately into the sinus venosus (Fig.1C).

The vascular system of 3 to 4-day-old embryos has changed dramatically. During the growth of the embryo the proximal parts of the left and right omphalomesenteric veins are translocated from their lateral positions (Fig.1C) to the midline of the embryo (Fig.2A). Near the confluence of the omphalomesenteric veins into the sinus venosus of the 4-day-old embryo, a vascular plexus has developed in the area of the liver diverticulum, developing from the ventral wall of the foregut (Figs.2B and 2C). Between the liver vasculature and the common cardinal veins, a small allantoic vein was also visualized. The plexiform common cardinal vein has enlarged compared to the 2-day-old embryo, but still consists of irregular, anastomosing endothelial tubes (Figs.2A and 2B). In this stage, a large ventral pharyngeal vein (Fig.2B), arising from the common cardinal vein, could be visualized. In earlier stages, this vessel was not visualized.



Figures 1A,B,C. Dorsal views of 2-day-old quail embryos. Figs.1A and 1B. The dorsal aortae (*da*) with the dorsal intersegmental arteries (*dia*), start to fuse (*arrows*). The dorsal aortae blend caudally into the omphalomesenteric arteries (*oa*). Note the segmental impressions of the somites between the *dia*'s. The common cardinal veins (*ccv*) drain through the omphalomesenteric veins (*ov*) into the sinus venosus. The dotted line indicates the level of the section in Fig.6A. Fig.1A: Bar = 1 mm; Fig.1B (enlargement of Fig.1A): Bar = 100 μm. Fig.1C. A late 2-day-old embryo. The plexiform omphalomesenteric veins (*ov*) and common cardinal veins (*ccv*) flow separately into the sinus venosus (*sv*). In the atrial segment (*A*) a smooth impression of the developing atrium septum (*arrow head*) is present. The heart tube is looped, with the outflow tract (*O*) right-sided. Leakage at the place of injection (*L*). Fig.1C: Bar = 100 μm. Fig.2A. 3-day-old quail embryo in a right-sided view. The venous system is greatly enlarged compared to figure 7. The posterior (*pcv*) and anterior (*acv*) cardinal veins communicate by several branches of the common cardinal veins (*ccv*) with the sinus venosus (*sv*). Both omphalomesenteric veins (*ov*) with the vitelline vein (*vv*), are located in the midline. Aortic sac (*as*); atrium (*A*); dorsal aorta (*da*); leakage at the place of injection (*L*); outflow tract (*O*). Bar = 1 mm. Figs.2B and 2C. 4-day-old quail embryos in right-sided views. The third (*III*), fourth (*IV*) and sixth (*VI*) pair of pharyngeal arch arteries are not completely filled with Mercox. The volume of the common cardinal vein (*ccv*) has increased, while most of their branches have disappeared. The liver vessels (*lv*) have developed from the omphalomesenteric veins (*ov*). Allantoic vein (*av*), atrium (*A*), ventral pharyngeal vein (*vpv*). Fig.2B: Bar = 1 mm; Fig.2C: Bar = 100 μm.



Figures 3A,B. Vascular casts of 5-day-old quail embryos.
Fig.3A. A right-sided view of the third (*III*), fourth (*IV*) and sixth (*VI*) pharyngeal arch arteries with a rudiment of the combined first and second arch artery (*arrows*). The pulmonary arteries, which are present in this stage as judged from MB1-staining, are not filled. The dotted line indicates the level of the section in fig 6B. Bar = 100 μm.
Fig.3B. A left-sided view of the caudal part of the single dorsal aorta (*da*) with the coeliac artery (*ca*), the unpaired omphalomesenteric artery (*oa*), the left and right dorsal intersegmental arteries (*dia*), and the subclavian artery (*sa*). The cast shows the arterial vessels (*m*) to the mesonephros arising from the lateral and ventral side of the dorsal aorta. The dotted line indicates the level of the section in Fig.6D. Bar = 1 mm.
Figure 4. Vascular cast of a 6-day-old quail embryo in a left ventral view. Between the pharyngeal arch arteries (*III*, *VI*) some branches of the ventral pharyngeal vein (*vpv*) are present, although hardly visible (*arrows*). At this stage the left fourth pharyngeal arch artery (*IV*) disappears, and therefore is not completely filled. The dotted line indicates the level of the section in Fig.6C. Bar = 100 μm.
Figures 5A,B. Dorsal view of a 7-day-old quail embryo. The transformed pharyngeal arch system consists of the right fourth (*IV*) and both sixth (*VI*) arteries. The coeliac (*ca*) and omphalomesenteric (*oa*) arteries arise from the right lateral aspect of the dorsal aorta (*da*). At both sides two to three arteries (*arrow heads*) vascularize the complete capillary plexus of the large metanephros (*m*), which drains into the posterior cardinal vein (*pcv*). Gonadic arteries (*ga*); iliac arteries (*ia*). The dorsal intersegmental arteries are incompletely casted (*arrows*). Fig.5A: Bar = 1 mm; Fig.5B (enlargement of the left metanephros of Fig.5A): Bar = 1 mm.

In the majority of the embryos, the pharyngeal arch system (Figs.2B, 3A and 4) was visualized, but in some cases one or several arch arteries were not or only incompletely filled (Figs.2A, 2B and 4). Before septation of the outflow tract of the heart it was impossible to fill the pulmonary system probably because of its high resistance with respect to the pharyngeal arch system (Fig.3A). In the 6-day-old embryo the ventral pharyngeal vein has extended considerably compared to the 4-day-old stage. Some of its tributaries nearly contact the pharyngeal arch arteries (Fig.4).

The unpaired omphalomesenteric and coeliac artery change their ventro-medial position in the 5-day-old embryo (Fig.3B) towards a right-lateral one with respect to the dorsal aorta in the 7-day-old embryo (Fig.5A). Another important event during these days is the differentiation of the mesonephric vasculature. The arterial supply to the mesonephros in the 5-day-old embryo, consists of many separate branches from the dorsal aorta (Fig.3B). In 6 and 7-day-old embryo's, the number of branches is reduced to three arteries at both sides of the dorsal aorta (Figs.5A and 5B). The mesonephros is drained by the posterior cardinal vein (Fig.5A).

Immunohistochemistry

Organogenesis in the stages studied, is attended by the development of an organ-specific vasculature. The blind-ending tributaries of the large arteries and veins in the vascular casts indicate the location of their development. Extending sprouts observed during development, e.g. the ventral pharyngeal veins (Figs.2B and 4), suggest that these endothelial-lined vessels are outgrowths from existing vessels. To examine whether such sprouts are complete replications of the lumenized part of a developing vessel or incomplete replications of an already existing lumenized vascular plexus, quail embryos of comparable stages were studied light microscopically with special attention to the plexus of endothelial cells and their precursors of the dorsal intersegmental vasculature, the aortic arch system and the mesonephric vasculature.

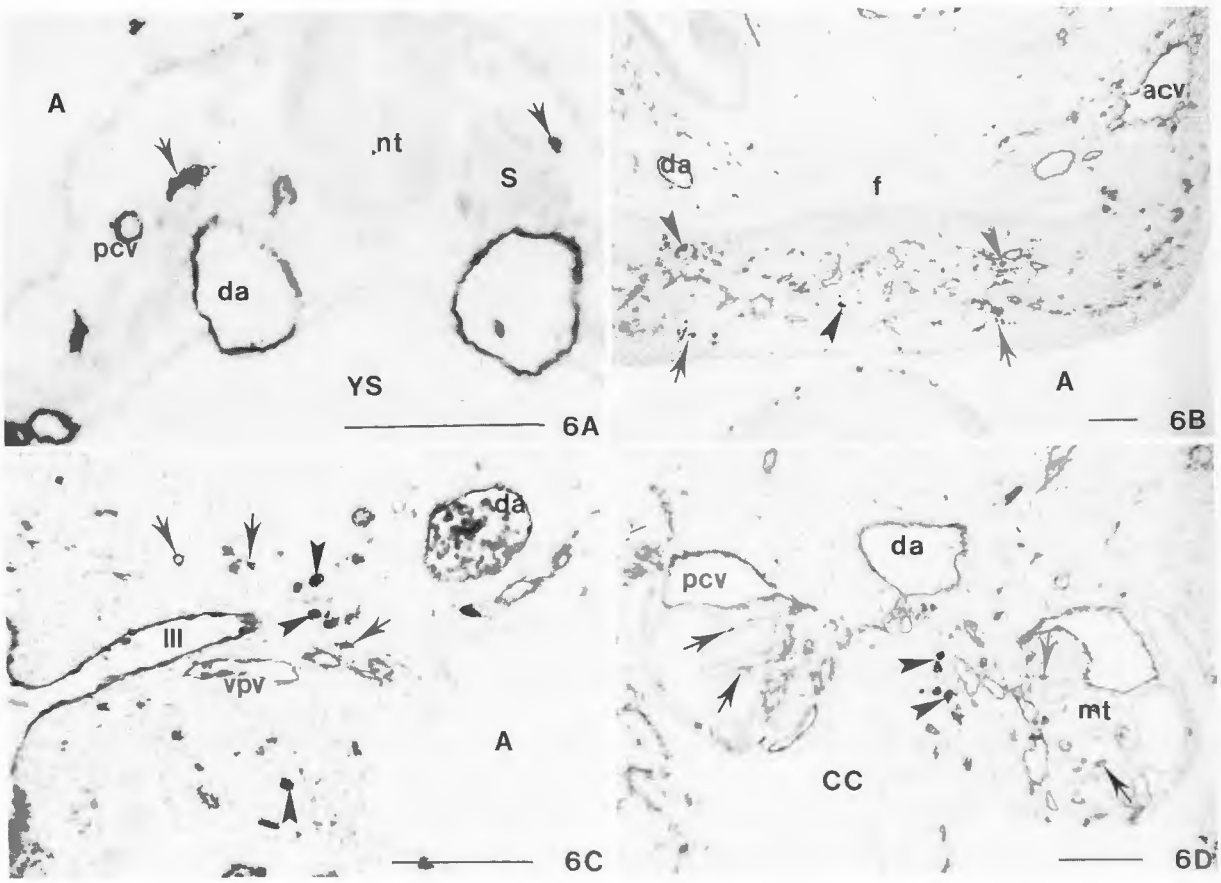
In 2-day-old embryos, a plexus of endothelial precursors is present in the paraxial mesoderm, forming the dorsal intersegmental vasculature and posterior cardinal veins (Fig.6A). The endothelial precursors, recognized by MB1, are elongated cells, that do not line a lumen. Usually they are connected to a number of like cells. They can be distinguished from the various hemopoietic stem cells, which are always round and larger (Figs.6B, 6C and 6D). The connections with the dorsal aortae and the developing posterior cardinal veins may contain already a small lumen, that are lined by endothelial cells. In subsequent stages of development the endothelial precursors align to form small lumenized vessels connecting the dorsal aortae and the posterior cardinal veins. At some places the lumen of the dorsal intersegmental vessels is still irregular.

In 2-day-old embryos the first and second pharyngeal arch arteries have developed from a plexus of endothelial precursors, located between the outflow tract of the heart and the dorsal aortae. The pharyngeal arch arteries are connected bilaterally with a plexus of endothelial precursors, which blends caudally into the ventral pharyngeal vein containing an irregular lumen. At the place of connection with the common cardinal vein, the ventral pharyngeal vein presents its widest lumen. The plexus remains in contact with the first and second pharyngeal arch arteries as well as later on with the more caudally developing arch arteries, but will lumenize over larger parts. In 5-day-old embryos the third, fourth and sixth pharyngeal arch arteries are endothelial-lined tubes, while the first and second pharyngeal arch arteries have branched into a second, but nevertheless lumenized plexus, located in the concomitant pharyngeal arches (Fig.6B). During this remodelling, the second plexus is still connected to the plexus of the ventral pharyngeal vein. In view of the complexity of the plexus in these pharyngeal arches, which now consists of lumenized and non-lumenized parts, it was impossible to assess whether a complete lumenized arterial-venous connection is present, allowing venous drainage in this stage. Other lumenized tributaries of the enlarged ventral pharyngeal vein in the 5-day-old embryo are still connected via a plexus of endothelial precursors with the third, fourth and sixth pharyngeal arch arteries (Fig.6C). At the place of connection, small lumenized sprouts can be present.

The central part of the mesonephric tubules of 4 and 5-day-old embryos has a lumenized vasculature situated between the dorsal aorta and the posterior cardinal vein (Fig.6D). Between the tubules, many endothelial precursors are present. In subsequent stages, the lumenized vessels will expand also between the tubules.

Discussion

In this study of 2 to 6-day-old quail embryos, the vascular system was injected with Mercox. The results show that the casting-technique is excellent to investigate the patterns and remodelling of the large embryonic vessels. It also yields detailed information on small vascular plexus, places of fusion between both the dorsal aortae and the metameric pattern of the intersomitic arteries. Impressions of endothelial nuclei, as often seen in adult material, which are more pronounced in arteries than in veins (Hodde and Nowell 1980, Kardon et al. 1982), were rarely found. An explanation could be the flexibility of the blood vessel wall, supported by the surrounding mesenchyme. In the stages studied, the tunica media is starting to differentiate. It only consists of a small amount of reticular fibers between up to 4 cell-layers (El-Maghraby and Gardner 1972, Hughes 1943), containing actin filaments (DeRuiter et al



Figures 6A,B,C,D. Light micrographs of transverse sections of quail embryos incubated with the MB1 antibody.
Fig.6A. Endothelial precursors at the location of the future dorsal intersegmental arteries (*arrows*) and the posterior cardinal veins (*pcv*) in the 2-day-old embryo (HH-stage 11^{+}); see Fig.1A. A small lumen is present close to the dorsal aortae and the developing posterior cardinal vein. Amniotic cavity (*A*), dorsal aortae (*da*) neural tube (*nt*), somite (*S*), yolk sac cavity (*YS*). Bar = 100 μm.
Fig.6B. Section (see fig 3A) through the second pharyngeal arch of a 5-day-old embryo. The formerly continuous second pharyngeal arch arteries are reorganized and form an extended irregular plexus of endothelial precursors in the arch. The arrows indicate the distal part of the plexus of the ventral pharyngeal veins. The hemopoietic stem cells (*arrow heads*) are round and large. Anterior cardinal vein (*acv*) and foregut (*f*). Bar = 100 μm.
Fig.6C. In the 5 to 6-day-old embryo the left ventral pharyngeal vein (*vpv*) (see Fig.4) has lumenized tributaries, which blend into a plexus of endothelial precursors (*arrows*). They contact the pharyngeal arch arteries (*III*). Bar = 100 μm.
Fig.6D. Section through the mesonephros of a 5-day-old embryo (see Fig.3B). The central part of the mesonephric vasculature has an irregular lumen. Between the mesonephric tubules (*mt*) endothelial precursors (*arrows*) are present. Coelomic cavity (*CC*) Bar = 100 μm.

1990B). It is evident that such a construction presents less resistance than an adult vessel wall.

In contrast with the adult artery and vein patterns, the embryonic vasculature is characterized by multiple afferent and efferent vessels to an organ. In addition, large arteries and veins may branch into a complex of anastomosing channels, like the pharyngeal arch arteries and the common cardinal veins. When these channels are not simultaneously filled with Mercox, it frequently hampers complete casting of all paralleling channels.

In adult (Aharinejad et al. 1989, Imada et al. 1987, Kardon et al. 1982, Murakami et al. 1989, Schraufnagel 1987, Schraufnagel 1989) and early postnatal material (Caduff et al. 1986), Mercox seems to be a suitable media to cast complete arterial-capillary-venous systems. The diameter of the smallest capillaries measured, deduced from the above cited literature, is about 3 to 4.5 μm. In our series of embryos, it is

remarkable that tributary vessels of large arteries and veins, as the dorsal intersegmental system, the vasculature in the pharyngeal arches and in the mesonephros, are not completely casted. Only short blind-ending branches with diameters larger than 20 μm, are present. In other investigations using Mercox in embryos of comparable stages (Bockman et al. 1989, Phillips et al. 1989, Sumida 1988), complete arterial-venous plexus were not casted either. In general, imperfect filling could be attributed to precocious polymerization of the Mercox. Even when the Mercox was not viscous or cured at the termination of our injections, the tributary vessels were not filled. Moreover, with the same technique, we are able to cast 3 μm capillaries in complete arterial-venous plexus of late fetal and postnatal chick and rats (DeRuiter unpublished data, VanGroningen et al. 1991). To explain this difference between adult and embryonic material the question arises whether the blind-ending branches are complete casts of developing lumenated tributary sprouts or the result of incomplete filling of an existing lumenized vascular system by a high peripheral resistance caused by an incomplete removal of the embryonic blood?

The blind-ending branches in the Mercox casts suggest that the organ-specific vasculature arises by budding and branching from pre-existing vessels. This mechanism is called angiogenesis (Coffin and Poole 1988; Poole and Coffin 1988, 1991; Risau 1991). A new sprout extends into the surrounding mesenchyme by mitosis of the migratory endothelial cells at its tip (Poole and Coffin 1989). The first example of angiogenesis in the embryo should be the development of the dorsal intersegmental arteries (Poole and Coffin 1991). Another theoretical concept involved in the formation of blood vessels is vasculogenesis. Blood vessels as the dorsal aorta, endocardial tube and cardinal vein, should be established by the adherence of in situ differentiating endothelial cells from the mesenchymal tissue (Poole and Coffin 1989, 1991; Risau 1991). Vasculogenesis seems to be restricted to the early embryonic development (Risau 1991) in an interaction between the mesodermal with the endodermal germ layer (Pardanaud et al. 1989), while angiogenesis may occur during the entire lifespan (Risau 1991).

Our serial sections of the quail embryos, incubated with the monoclonal antibody MB1, show that a plexus of endothelial precursors is already present in the mesenchyme of a developing organ before lumenized sprouts have arisen from an accompanying pre-existing artery and vein. After adherence of the endothelial precursors to a vessel wall, they are incorporated in the endothelial lining of the developing lumenized sprouts or even in the pre-existing artery or vein (Christ et al. 1990). Outgrowth from existing vessels into an endothelial precursor-free mesenchyme was not observed. Therefore our observations support the mechanism of vasculogenesis in tributary vascular systems of the dorsal aortae and cardinal veins instead of angiogenesis. This corresponds with the quail-chick transplantation experiments of Noden (1989, 1991) and Wilms and coworkers (1991), which shows that all embryonic mesoderm populations have angiogenic potentials. However, it is questionable whether the whole endothelial plexus is completely derived from in situ differentiation from the mesenchyme, or whether it develops by cell division of only a few early present endothelial precursors. Recent investigations (DeRuiter et al. 1990A; Gittenberger-de Groot et al. 1990) have shown that a two-dimensional, in situ differentiated horseshoe-shaped vascular network of a 4-somite quail embryo is remodeled by the growth of the embryo in such a way that it gives rise to the endocardium, pharyngeal arch arteries, dorsal aortae, and vitelline veins. During this remodelling, single as well as strands of endothelial cells (endothelial precursors) become situated between the newly-formed vessel systems. It has been shown that these precursor cells contribute in turn to the formation of the pharyngeal arch arteries, the pulmonary arteries and the ventral pharyngeal veins. In a comparable way, Poole and Coffin (1991) also described that endothelial precursors of the yolk sac vessels differentiating in the lateral splanchnic mesoderm, can cross towards the somatic mesoderm, contributing to the formation of the posterior cardinal veins. This implies that besides vessel formation by in situ differentiation of endothelial cells and/or by sprouts invaginating into a developing organ, single or strands of already present endothelial cells also contribute to the development of an organ-specific vasculature. These cells do not differentiate in situ in the developing organ, but originate from another pre-existing plexus.

Complete casting of the dorsal intersegmental vasculature in 2-day-old embryos as well as the connections between the ventral pharyngeal vein and pharyngeal arch arteries, was not possible, due to the lack of lumen of adequate size. During organogenesis more and more single endothelial precursors are incorporated into the enlarging sprouts and will line a lumen. After circulation has been established, the lumen of the new capillary plexus, present in the first and second pharyngeal arches as well as between the somites and in the mesonephros of 4 and 5-day-old embryos, is at first irregular and at places still interspersed with vascular parts mainly consisting of endothelial precursors. None of these capillary plexus could be completely filled with Mercox. This indicates that the resistance of a shortly before developed lumenized capillary plexus is probably too high for the viscous Mercox. This is supported by the vascular casts of the mesonephros. Until 5 days the mesonephric capillary plexus could not be casted completely. According to Patten (1971) the functional activity of the mesonephros is at its height from the fifth until about the eleventh day of incubation. The complete lumenized part of the

mesonephric vasculature of 6 and 7-day-old embryos could be easily casted, which suggests a reduction of the vascular resistance from the moment of functional activity of an organ. The review of Polgar and Weng (1979) on the development of the lung supports this hypothesis. The pulmonary vascular resistance is very high in mid-gestation, but decreases progressively to approximately one-twentieth of that value in term lambs. Moreover, they stated that large portions of the fetal pulmonary vascular bed is not perfused until birth. Therefore it can be concluded that Mercox is a suitable media to study the growth and remodelling of the large arteries and veins in the embryo, but complete filling of a developing tributary vascular system is usually hampered by the high vascular resistance caused by its irregular lumen. Immunohistochemistry, using anti-endothelium antibodies, is a powerful tool to fill in the gaps left by the incompletely casted irregular parts of the vascular system.

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Discussion with Reviewers

G. Burton: In view of the small diameter of the vessels to be casted did you consider using a medium of lower viscosity? What was the viscosity of the resin?

D.A. Steeber: You state that the incomplete filling of vessels, especially the capillary plexus, may be due to both high vascular resistance and the high viscosity of the Mercox resin. In some cases we have found that it was necessary to lower the viscosity of the Mercox resin, by diluting it with monomeric methylmethacrylate, to obtain complete casts. Have you tried lowering the viscosity?

Authors: We have not measured the viscosity of the Mercox CL-2B. The viscosity is the same (Viscosity: 32.37 cStoke at 20°) as measured by Weiger and coworkers (1986). We have diluted Mercox with methylmetacrylates, but in our hands we could not fill considerable larger parts of the vascular system. Moreover, in this study we worked with very small amounts of resin. It appeared that, in using such small amounts of Mercox in combination with methylmetacrylates, the quality of the casts is decreased, resulting in fragmented casts that could not be studied anymore in the SEM.

G. Burton: Could some of the variations seen in the casting of the pharyngeal arch vasculature in 4-day-old embryos be due to differences in the rate of development rather than incomplete filling?

Authors: This is not likely, because all the microseries of quail embryos between 90 hrs and 5 days show complete lumenized third, fourth and sixth pairs of pharyngeal arch arteries, indicating that this type of variability does not exist.

G. Burton: Throughout the paper the authors recognize the well-known problem of differentiating between capillary sprouts and unfilled vessels. Because the Mercox injections and the immunohistochemistry were not combined on the same tissue one still cannot be certain whether features seen on casts are sprouts or unfilled vessels.

Authors: A combination of both techniques would demonstrate beautifully the degree of filling with Mercox. From literature (Bockman et al. 1987; Phillips et al. 1989) and our own data, however, it is evident that the described developing vascular systems, which could not completely be injected with Mercox, can be injected without any problem with low-viscous Indian ink. This indicates that these developing vascular systems have a lumen, which is not accessible for media with a higher viscosity than that of e.g. blood.

G. Burton: Christofferson and Nilsson (1988) claimed that corrosion casts of capillary sprouts had a rather irregular pointed appearance due to swollen endothelial cells at the site of angiogenesis. Did your study support these findings?
Authors: In our casts this phenomenon of irregular ends of vascular sprouts is also noted, but it is questionable whether this is caused by swollen endothelial cells or by remaining blood cells, which are trapped within the blind-ending sprout.

B. Christ: Why did the authors not show the vessels of the limb buds? It is said that embryos have been examined up to the 7th day of incubation. In these stages well developed limb vessels do exist.
Authors: The stages studied indeed cover the period of limb bud formation and outgrowth. In the earliest stages of limb formation the vessels consist of a set of parallel arteries originating as segmental vessels from the aorta. Complete filling of all these vessels with the method used, led to variable results. One of the more successful injections in this respect is depicted in Figure 3B, in which a part of the subclavian artery is visible. Because a complete set of stages of limb bud development was hard to obtain, this was not further studied. In older stages the (partly) successfully casted limb buds were often removed, because they obscured the parts of the vasculature that were the main feature of this study.

G. Burton: Did you confirm from your serial sections that the fusion between the dorsal aortae indicated in figure 1B was indeed true fusion rather than extravasation of Mercox?
T. Poole: Do you have any idea of what is the driving force for the fusion of the two dorsal aortae?
Authors: The serial sections show unequivocally that both dorsal aortae are interconnected. It is questionable, however, whether both dorsal aortae fuse along their complete length. During the growth of the embryo the laterally situated dorsal aortae are relatively translocated towards the midline. During this process the mesenchymal cells originally located between the dorsal aortae disappear to leave only extracellular matrix material as a bridge between the two vessels. It is suggested that the endothelial cells at the site of branching act as a kind of zipper to fuse the dorsal aortae. This mechanism is maybe then comparable to e.g. the fusion of the neural walls during closure of the neural tube.

T. Poole: Are the intersomitic arteries more correctly referred to as plexuses or simple sprouts?
Authors: The intersomitic arteries, or dorsal intersegmental arteries, are small lumenized vessels at their origin from the dorsal aortae. They originate from a plexus, that initially consists of only a small number of endothelial precursors. Hereafter this plexus enlarges and gives rise to the dorsal intersegmental veins and posterior cardinal veins.

T. Poole: What sort of experimental approach might be used to determine the relative contributions of angiogenesis and vasculogenesis to organ vascularization?
Authors: The quail-chick chimaera seems to be a suitable technique to study the development of the vascular system. To allow for the incorporation of growth changes as an important factor, which influences the pattern formation of the developing vascular system, complete series of embryos with various survival times are needed to discriminate between active migration and relative translocations of the endothelial precursors or cells. 3-D reconstructions of some chimeras will probably be necessary.

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